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Growth and Physiology of Rickettsiae

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INTRODUCTION

The generic name "rickettsia" (uncapitalized) is often used very broadly and with little discrimination. A rickettsia is regarded as a minuscule organism, intermediate in size and properties between a bacterium and a virus, or any microorganism larger than a virus that can not be grown in lifeless media, or any small organism seen in insects and in other arthropods. It is not difficult to recognize the inadequacies of the above generalizations. (i) The difference between a bacterium and a virus is so great that no organism can be truly placed in an intermediate category (107). (ii) Moulder (74) and Hanks (45) have discussed the problems confronting, respectively, students of chlamydiae and of the noncultivable mycobacteria. These problems are quite different from each other and from those confronting the rickettsiologist (81). (iii) Organisms other than rickettsiae discovered in insects and ticks have seldom been studied in detail, but one such obligate intracellular bacterium, *Wolbachia persica*, for which there is a fair amount of information, appears to be quite different in morphology and physiology from rickettsiae (110, 111, 130).

What is then a meaningful definition of rickettsiae? This question can not be properly answered at this point, but possible definitions will emerge in the course of this review. For the purpose of orientation, it can be stated that the eighth edition of *Bergey's Manual of Determinative Bacteriology* (129) will list 10 species in the genus *Rickettsia* divided into three groups, namely, typhus, spotted fever, and scrub typhus rickettsiae. Although some investigators may prefer a somewhat different classification, there is wide agreement that these 10 species are sufficiently similar to be regarded as members of the same genus. The term "rickettsia" can also be extended to the monogeneric

genus *Coxiella*, which resembles rickettsiae in some respects and requires similar methodologies for investigation. This is not true of the trench fever agent (designated *Rochalimaea guintana* in the eighth edition of *Bergey's Manual*), which can be cultivated on relatively simple bacteriological media (77, 115). The inclusion of this organism among the rickettsiae is still of great value to the epidemiologist but of limited usefulness to the bacterial physiologist.

This review will be concerned only with bacteria belonging to the genera *Rickettsia* and *Coxiella*. It will be devoted to the properties which most clearly distinguish them from other organisms, namely growth and physiology. Other topics and other bacteria will be discussed only insofar as they contribute to the main purpose of this review.

STRUCTURE AND CHEMICAL COMPOSITION

Recent studies have yielded overwhelming evidence that the fine structure and chemical composition of rickettsia are entirely similar to those of other gram-negative bacteria (4, 5, 23, 54, 76, 79, 80, 89, 98, 108, 113, 114, 133, 136, 140, 143).

Members of the genus *Rickettsia* measure 0.3 to 0.5 μm in width but vary considerably in length. *R. prowazeki* is quite pleomorphic and its usual length is 2 to 4 μm, *R. rickettsi* measures approximately 2 μm, and *R. tsutsugamushi* measures 1.5 μm. *C. burneti* is somewhat smaller, 0.2 to 0.4 μm in width by 0.4 to 1.0 μm in length. In contrast to other rickettsiae, *C. burneti* undergoes an antigenic phase variation. Freshly isolated rickettsiae are in phase I. When passed serially in chicken embryos they are eventually replaced by phase II organisms. The cells of the two phases differ from each other in several respects, as discussed by Fiset and Ormsbee (35). Recently Wiebe et al. (136) isolated two types of *C. burneti* phase I cells on the basis of buoyant density. One type was small, compact, rod-shaped, and had a dense nucleoid. The other was somewhat larger, rounded, and had disperse nuclear filaments. Both types were viable and produced mixtures of both types when cultured separately.

Most electron micrographs of rickettsiae reveal a three-layered cell wall, but at high magnification it has been possible to resolve a five-layer architecture analogous to that described for *Escherichia coli* (4). The cell wall contains, in addition to sugars, amino sugars, and amino acids, muramic acid (89) and diaminopimelic acid (76, 143). Wisseman (140) has obtained evidence that rickettsiae have endotoxic activity, which suggests the presence of a lipopolysaccharide layer, typical of gram-negative bacteria. Teichoic acid, typical of gram-positive bacteria, was not detected. Capsular material surrounding the cell wall can be seen in electron micrographs of fresh preparations (4). There is also morphological as well as physiological evidence (79) of a cytoplasmic membrane.

The internal structure consists of electron-dense granules and fine strands which indicate the presence of ribosomes and deoxyribonucleic acid (DNA), respectively. Both ribonucleic acid (RNA) and DNA have been isolated from rickettsiae on numerous occasions. The RNA of *C. burneti* was subjected to electrophoretic analysis and RNA species were isolated with sedimentation constants of 4 to 5*S*, typical of transfer RNA, and 16 and 23*S*, typical of ribosomal RNA (113). The size of the DNA is comparable to that of other microorganisms of similar size (54). Recent studies (114) have permitted a detailed comparison of the molar percentages of guanine plus cytosine (% G + C) of the DNA of several species of rickettsiae. The rickettsiae of the typhus group (101, 114) have approximately 30% G + C, and those of the spotted fever group have 32.5% G + C. This difference is highly significant and indicates an early evolutionary separation (114). Previous investigators had reported somewhat higher G + C contents for *R. prowazeki* and *R. rickettsi* (91, 144). The % G + C of *C. burneti* was determined by several investigators and was found to be approximately 43 to 45 (70, 99, 100, 103).

Thus, the obligate intracellular parasitism of the rickettsiae is not reflected in unusual morphologic or macromolecular characteristics. It is true that some of the observations made are not readily explained. For example, Anderson et al. (5) described small electron-lucent spherical structures in the cytoplasm of *R. prowazeki*. These are not understood, and at present no particular significance can be attributed to them. An unusually high DNA:RNA ratio has been reported in a number of cases, but this is probably due to loss of RNA from resting or damaged rickettsiae (28). The low % G + C is believed by some (102) to be typical of intracellular parasites and other organisms that are subjected to natural background ratioactivity which favors low guanine content, but are protected from ultraviolet radiation exposure which favors low thymine content. This is a highly speculative view which requires confirmation. In general, the unbiased investigator would be hard put to explain the obligate

intracellular parasitism of rickettsiae on the basis of structure and chemical composition.

GROWTH

Rickettsiae, despite several elaborate attempts, have not been grown in the absence of host cells. They are most commonly cultivated in fertile hen eggs, tissue cultures, or small laboratory animals, and, in certain specialized cases, in arthropods. Eggs have been most useful for the production of large harvests for antigenic and metabolic studies and for the manufacture of vaccines. Tissue cultures are better suited, however, for detailed observations of host-parasite relationships. Laboratory animals are still widely used for primary isolations, infectivity assays in some cases, as well as for tests of virulence and immunogenicity. Mass cultivation of rickettsiae in arthropods is now only of historical interest, but because of their roles as primary or secondary hosts, arthropods should be regarded as potentially useful experimental hosts. For the purpose of this review only information derived from eggs and from cell cultures will be examined.

Cultivation in Eggs

The method introduced by Cox of growing rickettsiae in the yolk sac of chicken embryos is by far the most satisfactory (30, 31). Fertile hen eggs are highly susceptible to infection with established strains and, in most cases, with new isolates. The incidence of infection of eggs inoculated with the Madrid E strain of *R. prowazeki* with concentrations covering the mean infective dose (ID_{50}) range is in agreement with the assumption that only one infectious rickettsia is required for infection (124). The ratio of total particles, identifiable by microscopy, to particles successful in infecting a chicken embryo varies with the species of rickettsiae and with the physiological condition of the rickettsial preparation. Wike et al. (139) determined the ratio to be 50:1 for *R. typhi* and 300:1 for *R. rickettsi*. The infectivity of *C. burneti* is so high that the ratio is presumably close to 1:1. The instability of scrub typhus rickettsiae has discouraged investigators from conducting quantitative determinations of this nature with this organism.

Chicken embryos are usually inoculated during the fifth or sixth day of embryonic development and, because they must be harvested before hatching time, they offer a 12- or 13-day period for the growth of rickettsiae. When the inoculum is one or a few infectious units, this period is long enough for maximal growth of established strains of typhus rickettsiae but not of the other rickettsiae. To make sure that infection is not missed in some of the embryos, a second passage is required for the spotted fever rickettsiae and a third passage for *C. burneti*. With larger numbers of rickettsiae there appears to be a linear relationship between size of inoculum and mean survival time of the embryos (Fig. 1). For certain strains of typhus rickettsiae this relationship is highly reproducible and has been widely applied for the titration of rickettsiae by the single dilution method (17, 124). With Q fever rickettsiae the range of useful concentrations is small (82).

The optimal temperature for the growth of spotted fever rickettsiae is 33.5 C, a compromise between the optimum for the rickettsiae, which is probably about 32 C, and the minimum temperature generally required by embryos for their survival. For reasons that have not been

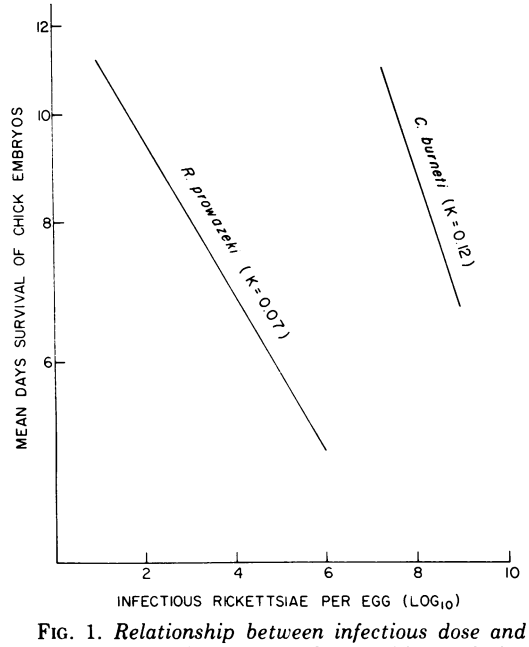

FIG. 1. *Relationship between infectious dose and mean time of survival of the embryos. This correlation is based on experiments with the Madrid E strain of R. prowazeki (124) and the Nine Mile strain of C. burneti (82). Both infectious rickettsiae and days of chicken embryo survival are plotted on logarithmic scales. When the concentration of R. prowazeki is reduced 10-fold the logarithm of the survival time of chicken embryos is increased by 0.07. By extrapolation it can be calculated that embryos inoculated with single rickettsiae survive for an average of 13.2 days. A 10-fold reduction in the concentration of C. burneti will increase the logarithm of the survival time by 0.12, but extrapolations to small numbers of infectious rickettsiae are not realistic.*

fully explored, the embryos are killed relatively early in the course of infection, but if the eggs are further incubated rickettsial multiplication in the yolk sacs continues for at least 2 days (109). The optimal temperature for the other rickettsiae is 35 C. In contrast to spotted fever rickettsiae, there is evidence that growth stops promptly when the embryos die and, in the case of typhus and scrub typhus rickettsiae, the titer declines rapidly after the death of the embryos (142). Optimal yields of infectious particles per egg are about 10^7 to 10^9 with most strains of *Rickettsia* and about 10^{11} with *Coxiella*. In terms of dry weight, harvests with members of the genus *Rickettsia*, measured after separation from host cell components, average from 0.05 to 0.5 mg/gm of yolk sac, *R. tsutsugamushi* yielding the smallest and the typhus rickettsiae the largest amounts. With *C. burneti*, yields of 1 to 2 mg/gm of yolk sac are not unusual.

The growth of rickettsiae in the yolk sac of chicken embryos has been amazingly useful in so many kinds of investigations (some more properly described in other sections) that its shortcomings have been obscured. Rickettsiae harvested from yolk sacs can be separated from host components only by relatively elaborate techniques which often result in damage to the microorganisms. As a model of rickettsial infection the yolk sac has rendered good service, but it has obvious limitations, which in some cases have been disregarded.

Host-Parasite Relationship as Observed in Cell Culture

General observations. Some of the events that take place in the infected yolk sac of the chicken embryo can be visualized by explanting the entodermal cells (128). The explants can be readily obtained from the avascular portion of the yolk sac of the 4-day-old embryo. The cells readily adhere to glass, and although they do not multiply to any extent, they expand into monolayers composed of a single cell type. These cells are highly vacuolated and have large, faintly staining cytoplasms that permit detailed cytological observation. Examples of cells infected with *R. prowazeki* and *C. burneti* illustrate the basic difference between these two microorganisms in their relationship to the host cell (Fig. 2). *R. prowazeki* is strictly confined to the cytoplasm, does not invade the vacuoles, and does not change the morphology of the host cell (120). *C. burneti*, on the other hand, multiplies in the vacuoles and during the progress of infection converts almost the entire cell into one large vacuole and compresses the nucleus and remaining cytoplasm towards the periphery (9, 132).

Spotted fever and scrub typhus rickettsiae have been studied in numerous cell types. Like typhus rickettsiae, they multiply primarily in the cytoplasm and not in the vacuoles, but they do display some important features of their own. Spotted fever rickettsiae, unlike other rickettsiae, are occasionally seen in the nucleus. The frequency of intranuclear invasion is greatest in mammalian cells, but they have been seen in the nuclei of avian and arthropod cells as well (24). Scrub typhus rickettsiae are strictly intracytoplasmic, but they usually aggregate in the region adjacent to the nucleus (22). *R. canada*, which in many respects, including DNA base composition (114), resembles the typhus rickettsiae (66), is occasionally seen in the nucleus (25).

Rickettsiae of each of the major groups have been grown in several types of both primary cultures and established cell lines, but comparative observations have been relatively few. Bozeman et al. (22) noted that most rickettsial species eventually formed dense masses in the cytoplasm of MB III mouse lymphosarcoma cells, but spotted fever rickettsiae remained loosely scattered even at the peak of infection. This difference was particularly striking when *R. tsutsugamushi* and *R. rickettsi* were compared in cultures of 14 pf rat fibroblasts (97). Besides major differences among rickettsial species, cell cultures in some cases reflected strain variation. Anderson et al. (5) showed that the growth of the Breinl strain of *R. prowazeki* in the BS-C-1 line of *Cercopithecus* kidney cells was more luxuriant than that of the avirulent Madrid E strain. Kordova et al. (58) obtained excellent multiplication of phase II *C. burneti* in L cells but very poor growth of phase I. Barker et al. (8) obtained equally satisfactory propagation of the Gilliam, Karp, and Kato strains of *R. tsutsugamushi* in BS-C-1 cells, but noted that the three strains produced different cytopathic effects. The reverse comparison of the same rickettsia in different types of cell culture was made by Kenyon et al. (53). Highest yields of *R. rickettsi* were obtained from duck embryo cells, followed by chicken embryo and Vero cells. Titers from L and human diploid WI-38 cells were considerably lower. There is no indication that other rickettsiae have the same degree of host cell specificity.

Plaque formation. Cloning of rickettsiae has been a goal of several investigators for some time, but it has become technically feasible only recently. Before the development of plaqu-

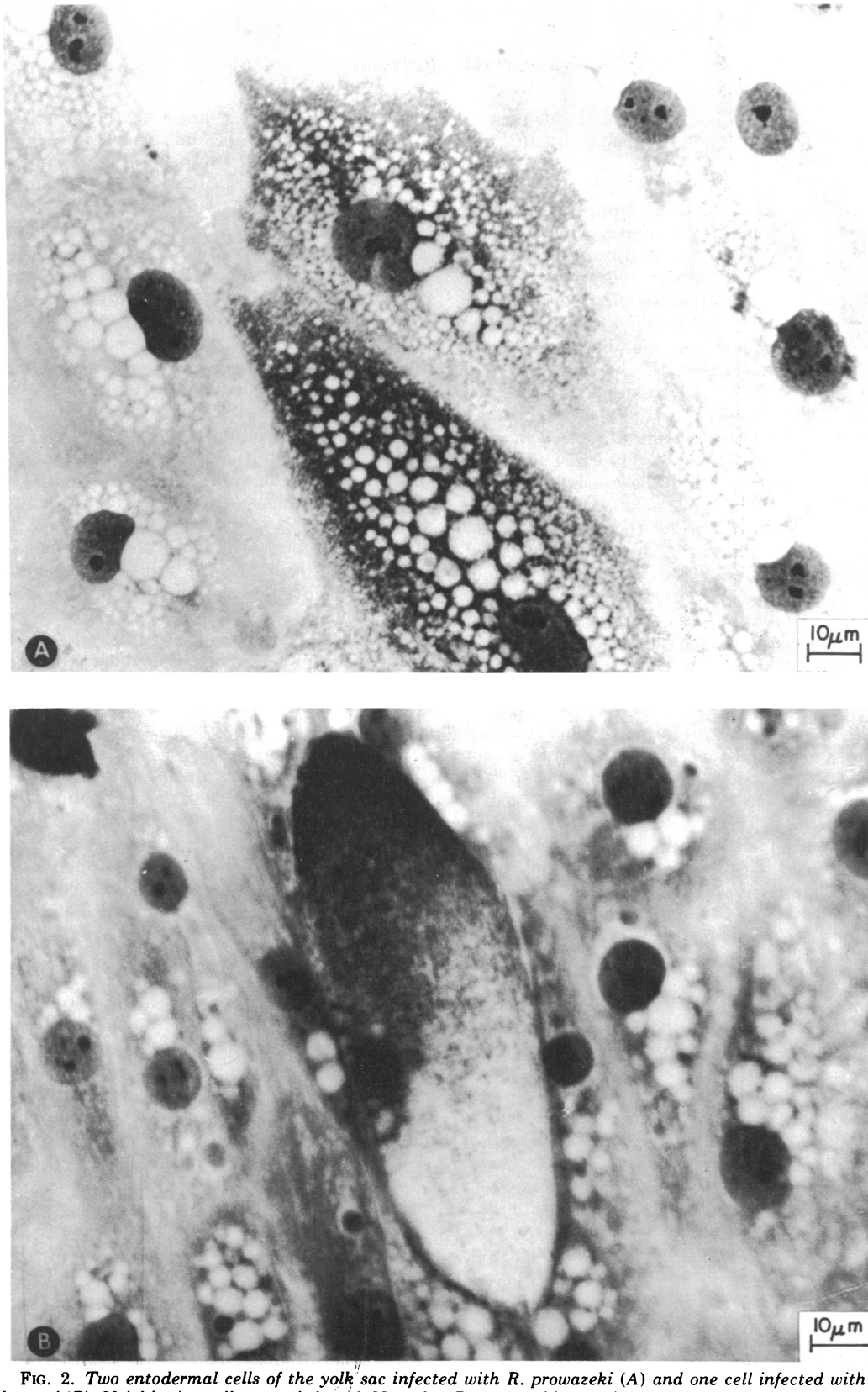

FIG. 2. *Two entodermal cells of the yolk sac infected with R. prowazeki (A) and one cell infected with C. burneti (B). Neighboring cells are uninfected. Note that R. prowazeki grows in the cytoplasm and does not alter its vacuolated appearance nor obviously affect the structure of the nucleus. C. burneti, on the other hand, grows in the vacuoles and eventually changes the entire host cell into one large vacuole and compresses the nucleus and remaining cytoplasm toward the periphery. A, from Weiss and Dressler (120); B, from Blackford (9).*

ing methods, it was possible to count infected cells or foci of infection in monolayer culture at low magnification (9, 120, 121, 132). Since rickettsiae migrated relatively slowly from cell to cell in these cultures, the counts reflected the concentration of the inoculum. This counting method, however, required staining of the cultures and thus had serious limitations.

The plaquing method was first developed by Kordova (57), who was not entirely satisfied with its reproducibility. Weinberg et al. (116) obtained consistent results with *R. rickettsi*, and McDade et al. (62, 63) extended the method to the other major rickettsial species. Primary chicken embryo monolayers were used in most of the above experiments. The chief technical difficulty which had to be surmounted was maintaining the monolayer under agar in good physiological condition for the required 8 to 17 days without stimulating growth to the extent that it would cover the plaques. Conditions affecting plaque formation were discussed by Wike et al. (139), who found among other things that suspension of the rickettsial inoculum in brain heart infusion broth and incubation of the cultures at 32 C was essential for satisfactory results.

When plaques are formed under optimal conditions, differences among the major groups of rickettsiae become apparent. The spotted fever rickettsiae require the shortest period to form plaques. They are first detected at 5 to 6 days but are more readily seen 2 or 3 days later, at which time they have a well-delineated margin and a diameter of 2 mm. The other rickettsiae develop smaller plaques, 1 mm in diameter (*R. canada* 0.75 mm), which sometimes have an indistinct perimeter. For best visualization, 10 to 12 days are required for typhus rickettsiae and for *R. canada*, 17 days for *R. tsutsugamushi*, and 8 to 10 days for *C. burneti*. The titers of most rickettsial preparations determined by the enumeration of plaques have been as high as, and in some cases slightly higher than, those obtained in eggs (63, 139). McDade and Gerone (62) reported in a brief study that the plaque titer of *R. tsutsugamushi* was somewhat higher than that obtained in mice, whereas the reverse was true of *C. burneti*. If these results can be confirmed, they would indicate that plaque titration is particularly useful for unstable organisms, such as *R. tsutsugamushi*, which require prompt contact with their host cells.

The usefulness of the plaquing technique for rickettsiae has been tested in various determinations such as antibiotic susceptibility (61), clone isolation (139), direct isolation from tick hemolymph or from guinea pig blood (137), absorption to host cells, and survival in various solutions (138, 139). Its full potential for kinetic and genetic studies has not yet been exploited.

Mechanism of penetration into host cells. It is essential to provide more than a casual contact between rickettsiae and host cells during the initial stage of infection. In entodermal cell cultures and certain other cell lines this is accomplished by centrifuging the rickettsiae onto the cells at 1,500 $\times$ *g* for 1 h at 20 C (121). Suspended cell cultures, such as MB III mouse lymphoblasts, are placed in a small volume of medium before infection with rickettsiae. After a period of absorption, usually 2 h at 37 C, medium is added to reestablish the original volume (22). Monolayers are generally overlaid with inoculum suspended in very small volumes and incubated at room temperature or at 37 C before the medium is added. If the monolayer is to receive an agar medium after the inoculum has been removed by washing, within limits the number of plaques increases with absorption time. It was shown by Wike et al. (139) that *R. rickettsi* continued to absorb at room temperature for as long as 4 h. Absorption time was greatly reduced, however, by centrifuging the cultures at 600 $\times$ *g* for 5 min.

There is good evidence that in addition to close contact necessary for absorption, the active participation of rickettsiae is required for penetration. This phenomenon has been studied by Cohn et al. (27) with *R. tsutsugamushi* penetrating into MB III mouse lymphoblasts. There are two other phenomena, studied most extensively by Bovarnick and Allen and their collaborators (3, 12-15, 105) with typhus rickettsiae, that need not result in penetration into suitable host cells, but most likely involve the same mechanism. One of these is mouse toxicity which is due to increased permeability of the endothelial cells of mice injected intravenously with high concentrations of rickettsiae. The other is hemolysis of rabbit or sheep erythrocytes elicited by rickettsiae. Both of these phenomena, but especially the latter, are well adapted to quantitative studies.

In general, only viable rickettsiae penetrate into host cells, are toxic for mice, or lyse red blood cells. For example, when *R. tsutsugamushi* is inactivated by heat (at 56 C for 5 min), by incubation with 0.1% Formalin, or by exposure to ultraviolet irradiation, it does not penetrate into host cells (27). Likewise, it was shown with typhus rickettsiae that viability, toxicity for mice, hemolytic activity, and respiration stimulated by glutamate are intimately associated. It is possible to selectively

destroy infectivity for the chicken embryo by ultraviolet irradiation (3), but all other attempts at selective inactivation have failed (3, 94).

Of the factors that affect penetration of rickettsiae into host cells, some influence the metabolic activity of the rickettsiae directly whereas others alter the rate of inactivation of extracellular rickettsiae. With a complete culture medium consisting of balanced salt solution (BSS), beef embryo extract, and horse serum, penetration of *R. tsutsugamushi* into MB III cells at 37 C proceeds linearly for 15 min and then the rate gradually declines. About 80% of the penetration takes place within 30 min, and after 120 min there is no further increase. Undoubtedly the stability of rickettsiae plays a role in the course of penetration, because there is an appreciable decline in infectivity of extracellular scrub typhus rickettsiae within 2 h at 37 C. When the complete medium is substituted by BSS alone, penetration of *R. tsutsugamushi* into MB III cells is reduced to about one half. When BSS is substituted by various combinations of sucrose and electrolytes, it can be shown that both monovalent and divalent metallic ions are required. The effect of the divalent ions appears to be relatively nonspecific; calcium and magnesium added singly serve as well as when added jointly, and they can be substituted by manganese, barium, or cadmium. One percent bovine serum albumin can substitute for beef extract and horse serum in the complete medium. This suggests that these proteins act primarily as stabilizing agents (27).

Of particular interest are the factors that most directly affect the metabolism of rickettsiae. Of a large number of substrates tested, only glutamic acid, glutamine, and a combination of α-ketoglutaric acid and aspartic acid (which yield glutamic acid in the presence of the proper transaminase) are effective in increasing the penetration rate of *R. tsutsugamushi* (27). Glutamic acid and glutamine are important substrates of typhus and spotted fever rickettsiae (21, 44, 91, 133), and this is probably the best evidence available that these compounds play similar roles in scrub typhus rickettsiae. Although the primary effect is most likely on the metabolism of the rickettsiae leading to cell penetration, it was shown in the same series of experiments that the survival of the rickettsiae in the absence of host cells was increased by glutamate (27).

Since the oxidation of glutamate by rickettsiae leads to the formation of adenosine triphosphate (ATP) (10), the above-described results suggest that rickettsiae must be capable of carrying out energy-yielding metabolism in order to penetrate into host cells. There is considerable evidence to support this suggestion for scrub typhus as well as for other rickettsiae. Penetration of *R. tsutsugamushi* is greatly reduced by various metabolic inhibitors such as 2,4-dinitrophenol or cyanide in the presence or absence of glutamate. Chloramphenicol, which is an effective inhibitor of the growth of rickettsiae (50), has no effect on penetration (27). Information for the other rickettsiae is of a more indirect nature. Wike et al. (138) showed that the inclusion of glutamate in suspending media increased the number of plaques formed by *R. rickettsi* and *R. typhi*. Pyruvate, an important substrate of *C. burneti* (84), increases the number of foci of infection in entodermal cell cultures (9). Glutamate also enhances the lysis of erythrocytes by typhus rickettsiae, provided the rickettsiae are not damaged. When the rickettsiae are severely damaged but not completely inactivated, ATP must be furnished (19).

It is well to remember that an active mode of penetration into host cells is not common among pathogenic bacteria. Chlamydiae are passively engulfed into vacuoles where they multiply (37), and neither viable nor nonviable *E. coli* or *Staphylococcus aureus* penetrate or are taken up by MB III cells under conditions favorable to scrub typhus rickettsiae (27).

Intracellular multiplication. There is overwhelming evidence from observations (too numerous to be cited) of stained smears or sections derived from infected yolk sacs, tissue cultures, laboratory animals, and arthropods as well as from electron microscopy that members of the genus *Rickettsia* multiply by transverse binary fission. Schaechter et al. (97) examined living cultures of 14 pf rat fibroblasts infected with *R. rickettsi* by phase microscopy and recorded this phenomenon photomicrographically on three separate occasions. His observations were confirmed and extended to other rickettsiae and host cells by Kokorin (55). Transverse binary fission appears to be the only mechanism of multiplication in the genus *Rickettsia*. The existence of a development cycle or the formation of inclusions, as in the case of chlamydiae, has been suggested in many instances but has never been substantiated. These suggestions were made most frequently with *R. tsutsugamushi*, which has a tendency to form aggregates near the host cell nucleus.

Light and electron microscopy observations have clearly indicated that *C. burneti* also multiplies by transverse binary fission. Kordova (56), however, presented evidence that binary fission of this organism is preceded by the

formation of a filterable form. Although Kordova's experiments were carefully executed, she left a number of questions unanswered, such as the size, chemical nature, and infectivity titers of these filterable forms. Until her work is confirmed in another laboratory and the nature of these particles is better defined, this hypothesis must be held in abeyance.

Rickettsiae appear to grow best in well-nourished cells. When *R. tsutsugamushi* was grown in MB III or L cells, an increase in titer occurred only in complete medium containing serum. In Eagle medium without serum the titer remained approximately constant, but when any one of the major constituents of Eagle medium was omitted, the titer declined rapidly (49). Except for the above findings and other casual observations, e.g., that in some cases calf serum is more satisfactory than fetal calf serum and that an acid pH is detrimental (131), the information on the nutritional requirements of rickettsiae is meager. An approach which shows considerable promise, involving the use of selective inhibitors of eukaryotic metabolism and labeled compounds, has not yet been exploited to study the details of nutrition.

Although rickettsiae require well-nourished host cells, the host cells need not multiply. It was shown that typhus rickettsiae propagated in entodermal cells previously exposed to doses of cobalt-60 irradiation of 100,000 R and in some cases as much as 300,000 R (120). Smaller doses, 3000 to 5000 R, which elicit giant cell formation but do not interfere with the adherence of the cells to the surface of the culture vessel, are used quite frequently with chlamydiae and rickettsiae (41, 131). Although it is not certain that irradiation increases the susceptibility of the host cells to rickettsiae, it simplifies the design of certain quantitative experiments by preventing unlimited host cell multiplication. Some of the experiments with *R. tsutsugamushi* were done with cells inhibited by colchicine (22). In these experiments the host cell is treated simply as a temporary, convenient microenvironment for the growth of rickettsiae.

Release from cells. The view that the cell infected with rickettsiae fills up with organisms and finally bursts and releases them into the medium is an oversimplification. This may occur with irradiated cells infected with typhus rickettsiae since, shortly after the peak of infection has been reached, virtually all host metabolic activity suddenly ceases (131). This phenomenon has not been studied morphologically, however. Careful observations on release of *R. rickettsi* and *R. tsutsugamushi* from the 14 pf cell line of rat fibroblasts were made by Schaechter et al. (97). Rickettsiae are sometimes trapped in microfibrillar structures protruding from the edge of the cell. When the microfibrils retract they either carry the organisms back into the cytoplasm or release them into the extracellular fluid. *C. burneti* propagates in vacuoles which gradually become filled with organisms. As the cultures age the number of organisms in the vacuoles decrease, suggesting that the rickettsiae are released over a variable period of time rather than suddenly (132).

There is also considerable morphological evidence that the rickettsiae are often released into neighboring cells. In experiments with monolayers of chick entodermal cells (120, 132), the count of foci of infection with *C. burneti* and *R. prowazeki* did not change appreciably as the infections aged from 5 to 10 days, even though the cells were not overlaid with agar. Each focus of infection consisted of one or more heavily infected cells and an increasing number of neighboring cells that were lightly infected. There was little evidence that new foci of infection were established at a distance from the older foci.

The mechanism of release of rickettsiae from host cells requires more careful attention. Many types of cells can be shown to be susceptible to infection and to be capable of supporting moderate growth, but relatively few cells release the rickettsiae in large numbers in a predictable manner. Good harvests of rickettsiae require such a release and prompt harvest, before the organisms lose their viability in the extracellular environment.

Effect on the host cell. In a study of the interaction of L cells and *Chlamydia psittaci*, Friis (37) showed that chlamydiae inactivated by heat or neutralized by antiserum were rapidly digested by the lysosomes. Viable chlamydiae, on the other hand, remained separated from the lysosomes and eventually multiplied in the vacuoles. He concluded that chlamydiae must possess a mechanism which prevents lysosomal activation. A similar mechanism may exist in *C. burneti*, which, like chlamydiae, multiplies in the vacuoles. It is conceivable that the disappearance of phase I in L cell and the rapid growth of phase II, noted by Kordova et al. (58), is due to a difference in interaction with L cell lysosomes. Members of the genus *Rickettsia* may bypass the action of the lysosomes by their active penetration into the cytoplasm and avoidance of the vacuoles.

Because rickettsial infection can be readily recognized by ordinary light microscopy, little attention has been paid to cytopathic effects demonstrable at relatively low magnification.

BS-C-1 cells infected with the Gilliam strain of *R. tsutsugamushi* were reported to be predominantly rounded, swollen globular in appearance, and those infected with the Karp strain were shrunken with tendency towards pyknosis and spindling, whereas the Kato strain induced areas of destruction in the sheet (8). *R. typhi* propagated in L cells produced little effect on the general appearance of the host cells until the cycle of growth was completed. *R. akari*, on the other hand, led to a number of obvious changes early in the course of infection. At 3 days, while growth was still proceeding rapidly, about half of the host cells had been released from the surface of the flask and those remaining were rounded or crenated. The above changes were reflected in the ability of the host cells to incorporate thymidine during the period of most rapid rickettsial growth—an observation facilitated by the failure of rickettsiae to take up this compound. *R. typhi* reduced host thymidine incorporation only to a moderate extent, whereas *R. akari* reduced it almost completely (131). The effect of *R. akari* appears to be analogous to that of chlamydiae which were shown to inhibit the thymidine kinase activity of their L host cells (60).

The above-described observations just serve to illustrate that the relationship of rickettsiae to their host cells may vary. Our knowledge is too limited to allow us to outline a basic pattern of interaction and to attribute an evolutionary significance to deviations from this pattern.

Inhibition of Growth and Drug Resistance

p-Aminobenzoic acid. Rickettsiae are unaffected by the sulfonamides. Most of them are inhibited, instead, by *p*-aminobenzoic acid (*p*AB) (104), which in other microorganisms acts as an antagonist of sulfonamide inhibition. This property is unusual but not unique. It has been encountered in *Rochalimaea quintana* (J. William Vinson, personal communication), *Wolbachia persica* (111), and in an isolate of *E. coli* (33). *Mycobacterium tuberculosis* is inhibited by a related compound, *p*-aminosalicylic acid, but this compound like the sulfonamides interferes with *p*AB metabolism (59, 145). The inhibition of rickettsiae by *p*AB has been demonstrated under a variety of conditions with newly acquired as well as old laboratory strains and in natural infection of man. Most of the species of rickettsiae are affected by *p*AB, but the susceptibility of *R. tsutsugamushi* is of a lower order and *C. burneti* is not affected (104). Although the therapeutic use of *p*AB is now only of historic interest, the study of this curious inhibition may contribute to the understanding of the physiology of rickettsiae.

The inhibition of rickettsiae by *p*AB can easily be demonstrated in eggs. When the inoculum of rickettsiae is sufficient to kill untreated embryos in 6 to 8 days, 1 mg of *p*AB, injected the previous day, delays the logarithmic growth of rickettsiae by 2 days and embryo death by 5 days. The rickettsial titer in the eggs at the time of embryo death is the same as in the controls (124). *p*AB has also been used in a number of in vitro tests, but in no instance has an effect been demonstrated on an extracellular activity of intact rickettsiae (18). Guardiola et al. (42, 43) showed that *p*AB reacts chemically with nicotinamide adenine diphosphate (NAD) and prevents malic dehydrogenase activity of *C. burneti* and *R. prowazeki* cell extracts. These results are thought provoking because *R. prowazeki* under certain conditions loses endogenous NAD (12, 14), but they do not explain why only the growth of typhus rickettsiae and very few other microorganisms is affected by *p*AB.

Davis (33) isolated a strain of *E. coli* that required both *p*-amino- and *p*-hydroxybenzoic acids, which furnished a useful model for the explanation of events in rickettsiae. Excess *p*AB interfered with the utilization of *p*-hydroxybenzoic acid (*p*HB), but the competition between the two metabolites was not symmetrical. Even large concentrations of *p*HB were not inhibitory. J. C. Snyder and B. D. Davis (Fed. Proc. **10**:419, 1951) applied this information to rickettsiae and showed that the inhibition of *p*AB could be competitively reversed by *p*HB. The minimal *p*HB:*p*AB ratio required for reversal was 1:10. Similar results were reported by Takemori and Kitaoka (112). Other explanations of the rickettsiostatic effect of *p*AB have been offered, namely that sulfonamides by some unknown mechanism favor the growth of rickettsiae or that *p*AB enhances the respiration of the host cells and this is deleterious to the rickettsiae, but these explanations have been disproven.

Weiss et al. (124–126) passed the Madrid E strain of *R. prowazeki* serially in eggs in the presence of concentrations of *p*AB that were slightly inhibitory, and they isolated three strains that were resistant to 1, 3, and 10 mg of *p*AB per egg, respectively. The increased resistance to *p*AB was accompanied by an increased susceptibility to salicylic and acetylsalicylic acids. The inhibition of acetylsalicylic acid (aspirin) was competitively reversed by *p*AB. The role of *p*AB thus changed from inhibitor to antagonist of inhibition. These re-

sults suggest that rickettsiae, like the strain of *E. coli* isolated by Davis, required both *p*HB and *p*AB. The requirement for *p*AB, however, was masked by its interference with the metabolism of *p*HB. When the drug-resistant rickettsiae were exposed to an inhibitory concentration of acetylsalicylic acid plus sufficient *p*AB for complete reversal plus increasing amounts of *p*HB, inhibition of growth again appeared: *p*HB reversed the action of *p*AB, not in its role as inhibitor, but in its role as an antagonist of inhibition. This interaction among the three compounds can only be explained by assuming that it occurs at two different sites in the rickettsial cell, presumably at the site of specific transport and at the enzymatic site. Only one change needs to have occurred in the resistant strains, namely a shift either in relative permease activity or in relative requirements for *p*HB and *p*AB. A diagram which attempts to explain the interaction of the three drugs is presented in Fig. 3. These studies were not continued because the selection of mutant strains of rickettsiae is time consuming and the results were not easily applicable to an understanding of other properties of rickettsiae.

Antibiotics. Penicillin and streptomycin inhibit the growth of rickettsiae to a small extent and are of no therapeutic value (104). Their effect can not be discounted, however, when rickettsiae are grown in cell culture. When the medium contains 60 μg of penicillin and 20 μg of streptomycin per ml, only *R. tsutsugamushi* can be cultivated. The other rickettsiae are materially affected by these concentrations of antibiotics, and the numbers of rickettsiae that can be recognized in infected cells decrease almost to the vanishing point within a few days (22). It was also shown that a mixture of 60 μg of penicillin and 50 μg of streptomycin per ml prevents plaque formation by *R. rickettsi*, except for pinpoint plaques appearing with very high concentrations of rickettsiae (116).

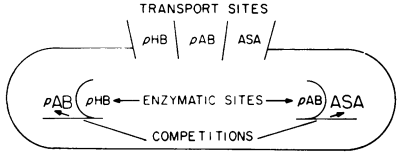

FIG. 3. *Attempt to represent the action of pHB, pAB, and acetylsalicylic acid (ASA) on rickettsiae. Competition may occur between two contiguous sites at the transport sites or between pHB and pAB or between pAB and ASA at the enzymatic sites. When the parent strain (pAB susceptible, ASA resistant) becomes pAB resistant (ASA susceptible), only one change needs to have occurred. pAB permease activity may be reduced, furnishing less pAB for competition with pHB utilization, but becoming more susceptible to transport competition with ASA. Conversely, the permeases may remain the same, but the relative enzymatic requirements may shift in favor of pAB. Thus less pAB remains to compete with pHB metabolism, but pAB metabolism becomes more susceptible to competition with ASA (126).*

Only with the advent of the "broad-range" antibiotics did chemotherapy of rickettsial infection become eminently efficacious. A detailed quantitative study of the effect of four antibiotics on the growth of four species of rickettsiae in eggs was carried out by Ormsbee et al. (83) and is summarized in Table 1. The tetracycline compounds are the most uniformly effective. The inhibitory dose of oxytetracycline is about one-eighth that of chlortetracycline. There is recent evidence (C. L. Wisseman Jr., personal communication) that the tetracycline compound doxycycline is even more effective than oxytetracycline in natural typhus infection of man. Chloramphenicol is effective against the species of the genus *Rickettsia* in doses about three times greater than those needed for chlortetracycline but, surprisingly, it is relatively ineffective against *C. burneti* (Table 1). The action of erythromycin is quite variable: it is highly inhibitory of *R. prowazeki*, has a moderate effect on two rickettsiae of the spotted fever group, and has only a slight effect on *C. burneti*. Experiments with *R. tsutsugamushi* (7, 51) indicate that the susceptibility of this rickettsia to the tetracycline compounds and to chloramphenicol is comparable to that of the other species of the genus *Rickettsia*.

When rickettsiae are exposed to the tetracycline compounds directly (105) or in tissue culture during adsorption (27), they are inacti-

TABLE 1. *Concentration of antibiotic causing a delay of 4 days in mean death time of infected chicken embryos*[a]

Organism	Antibiotic (μmol/egg)			
	Chloramphenicol	Chlortetracycline	Oxytetracycline	Erythromycin
Rickettsia rickettsi	0.71	0.25	0.028	0.33
R. akari	0.64	0.25	0.038	0.74
R. prowazeki	0.83	0.22	0.036	0.032
Coxiella burneti	5.90	0.28	0.032	>3.0

[a] Adapted from a more extensive presentation by Ormsbee et al. (83). This work confirms and extends the results obtained by Jackson (51). The antibiotics were administered 24 h after inoculation of the embryos with the rickettsiae. Slightly different results can be expected when the antibiotics are injected just before rickettsial inoculation.

vated at a relatively rapid rate. This does not happen when the tetracycline compounds are added after infection has been established (7), nor does it happen with chloramphenicol (50). The action of the latter antibiotic more closely resembles that of a metabolic inhibitor. Hopps et al. (50) exposed L cells infected with *R. tsutsugamushi* to the highest concentration of chloramphenicol tolerated by the L cells in long-term experiments, namely 5 μg/ml. When the infected cells were incubated at 35 C the number of rickettsiae decreased, but complete sterilization of the cultures did not occur until about 3 weeks of incubation. Up to that time the presence of rickettsiae could be demonstrated by subinoculation into mice or simply by removing the antibiotic from the cultures. Large numbers of rickettsiae reappeared in the host cells a week or two after the removal of chloramphenicol. It is interesting to note that infected L cells suspended in a nonprotein medium without chloramphenicol lose their rickettsiae more rapidly than those that are cultivated in a complete medium containing 5 μg of chloramphenicol per ml (49, 50). These results indicate that *R. tsutsugamushi*, which is highly unstable in an extracellular environment, is quite stable within well-nourished host cells even when it is not able to multiply.

Attempts to isolate antibiotic-resistant mutants in the laboratory were made by Weiss and Dressler (117, 122, 123) with the Madrid E strain of *R. prowazeki*. After 40 serial passages in the presence of increasing amounts of chloramphenicol and one limit-dilution passage, a substrain was isolated which appeared to be the result of a two-step change, each involving a twofold increase in antibiotic requirement for a given degree of inhibition. Within the limits of these experiments it appeared difficult to go beyond the second step in resistance. Antibiotic resistance was not lost after 10 drugless egg passages at high concentration, which suggests that the susceptible strain did not have a selective advantage over the resistant strain as is the case with some bacteria. Resistance to chloramphenicol was accompanied by a corresponding increase in resistance to the related compound thiocymetin. In another series of experiments an erythromycin-resistant strain was isolated. The change probably occurred during the first passage and involved complete resistance to this antibiotic. The parent strain was highly susceptible to erythromycin: 0.01 mg per egg had a demonstrable inhibitory effect, and although the antibiotic had no in vitro activity on the rickettsiae, doses of 1 mg per egg sterilized the inoculated eggs. The resistant strain did not appear to be affected by doses as large as 2 mg per egg. This level of resistance was even greater than the one encountered with "wild" strains of *C. burneti*. Resistance to erythromycin extended to the chemically related antibiotics carbomycin and oleandomycin. Attempts to isolate a tetracycline-resistant strain were unsuccessful. Several attempts have been made by using strains resistant to *p*-amino benzoic acid, chloramphenicol, and erythromycin to demonstrate acquisition of drug resistance by genetic transfer in eggs or entodermal cell cultures. These experiments have not been successful (123).

Antibiotic-resistant strains of rickettsiae have not been isolated in nature.

METABOLISM

The discovery by Bovarnick and Snyder in 1949 (21) that typhus rickettsiae consume oxygen and produce carbon dioxide in the presence of glutamate represented a milestone in the study of rickettsiae. The finding has been repeatedly confirmed and extended. It is difficult, however, to present a single outline of the metabolism of rickettsiae. Comparative studies have been few and two main approaches have been followed. Bovarnick and some of the other investigators who worked primarily with thyphus rickettsiae used viable cells. This approach has the advantage that it studies the physiology of the rickettsial cells rather than just the presence or absence of enzymes. It has the shortcoming that the rickettsiae, subjected to a long series of steps designed to separate them from host enzymes, are injured to a lesser or greater extent and are not true representatives of intact resting cells. Paretsky and his collaborators, who studied *C. burneti*, chose to disrupt the rickettsial cells and thus eliminate the problems of transport and possibly reduce the effect of cell injury. The third approach, the study of the metabolism of rickettsiae multiplying inside host cells by the judicious use of radioisotopes and metabolic inhibitors, has been used only recently. Ideally all these methods should be used on the same rickettsia, but since this has not been done, typhus rickettsiae and *C. burneti* will be treated separately. The relatively few experiments done with spotted fever rickettsiae will be integrated with the former. The metabolism of scrub typhus rickettsiae was not studied except indirectly as described in the section on cell culture.

Typhus and Spotted Fever Rickettsiae

Substrates that stimulate respiration. Respiration of rickettsiae in the absence of added substrate is negligible, but it is stimulated by a

moderate number of substrates, most vigorously by glutamate (21). The rate of utilization of this substrate is considerably higher in typhus than in spotted fever rickettsiae and varies widely from preparation to preparation. In terms of oxygen uptake per milligram of protein at 32 to 34 C, it seldom exceeds 1.5 μmol/h and often is much lower, and it correlates well with other biological activities such as infectivity for the chicken embryo, mouse toxicity, or hemolytic activity (13, 14, 48, 133).

The second most stimulating substrate of the respiration of both typhus and spotted fever rickettsiae is glutamine (44, 133). The ratio between respiration stimulated by glutamate and glutamine is approximately 3:2. It was shown by Hahn et al. (44) that glutamine is deamidated by *R. typhi* to glutamate and that this is probably the only pathway by which glutamine is metabolized by resting (extracellular) rickettsiae. Since glutamine is an important metabolite of cultivated mammalian cells, required in concentration second only to glucose, this reaction deserves further investigation. It is not known, for example, whether the more rapid stimulation of respiration by glutamate is due to more rapid transport of this compound or to the fact that the deamidation of glutamine is the rate-limiting reaction.

Other substrates stimulate the respiration of rickettsiae to a much lesser extent and these reactions can be demonstrated by conventional manometric techniques only with unusually active preparations. Among them are pyruvate and the dicarboxylic acid intermediates of the citric acid cycle (141). The same is true of the spotted fever rickettsiae (91).

Glucose, glucose-6-phosphate, lactate, sucrose, and the naturally occurring amino acids, except for glutamate, do not stimulate respiration (21). The failure of rickettsiae to catabolize glucose has been confirmed by sensitive radioisotope techniques which measure CO_2 production (85, 134). It is not known whether this is due to lack of the proper permeases, or one or two key glycolytic enzymes, or the entire glycolytic system (see Q fever rickettsiae below).

The evolutionary significance of the ability of rickettsiae to catabolize glutamate and their inability to utilize glucose is difficult to assess, but a few cautious comparisons can be made with other organisms. Both *Neisseria meningitidis* (69) and *Brucella abortus* (38, 39) metabolize glutamate more rapidly than any other substrate, utilizing it for growth and as a source of energy. The rate of O_2 uptake of a typical suspension of resting cells of *N. meningitidis* is about 10 μmol per h per mg of protein, or 6 to 12 times greater than that of a comparable preparation of rickettsiae (69). The rate for *B. abortus* is lower than that of *N. meningitidis*, and it was shown by Dasinger and Wilson (32) that a virulent strain of *B. abortus* oxidizes glutamate at about half the rate (1.5–2.0 μmol of O_2 uptake per h per mg of protein) of an avirulent strain. This is possibly related to the finding by Freeman et al. (36) that avirulent brucellae can not be cultivated in guinea pig monocytes, not because they do not have the ability to grow in them, but because they destroy them too rapidly. If experience with brucellae is applicable to rickettsiae and manometric experiments reflect intracellular events, the rate at which rickettsiae remove glutamate plus glutamine from the host cell for their own use is probably about as high as can be expected. A higher rate would lead to the destruction of the intracellular environment.

Pathway of glutamate utilization. Glutamate metabolism of resting rickettsiae does not appear to be different from that of other microorganisms even though some of the reactions require further elucidation. Three end products have been recognized: ammonia, carbon dioxide, and aspartate.

The amino group of some of the glutamate is released as ammonia, but Bovarnick and Miller (16) have shown that most of the amino group is transferred to oxaloacetate with the formation of aspartate. The enzyme involved in this reaction, glutamate-oxaloacetate transaminase, is one of the most stable rickettsial enzymes. Rapid freezing and thawing of rickettsial suspensions, which destroys most of the oxidative enzymes, greatly enhances this activity. The addition of pyridoxal phosphate is not required, suggesting that the enzyme remains saturated with its cofactor (48). The transaminase may also catalyze the reverse reaction: respiration in the presence of α-ketoglutarate plus aspartate is always much greater than the sum of the respirations that take place when the two substrates are added singly. Presumably, there is enough extracellular enzyme in the cell suspensions to elicit the formation of glutamate, which is then rapidly transported into the cell and oxidized. Bovarnick and Miller (16) attributed the origin of the extracellular enzyme to the disrupted cell which is always present in a rickettsial suspension. The possibility should not be overlooked, however, that the transaminase is associated with a surface structure.

Carbon dioxide is produced from all carbon atoms of glutamate, although it is not certain that glutamate is completely oxidized (96). α-Ketoglutarate, succinate, fumarate, malate,

oxaloacetate, and pyruvate are intermediate products of the reaction and do not accumulate without specific inhibitors (141). Pyruvate is degraded with production of CO_2 from all three carbons (133). Attempts to demonstrate the formation of citric acid and other tricarboxylic acids have not been successful, but this might have been due to technical difficulties rather than to the total absence of the corresponding enzymes in rickettsiae (141).

Hayes et al. (46) used the sensitive spectrophotometric method of Chance (26) to study electron transport in suspensions of intact rickettsiae. Their studies indicated that *R. typhi* has a flavine enzyme-iron-cytochrome system which probably includes cytochromes a_1 and b_1. Although the terminal respiratory enzymes are comparable to those of other bacteria, the specific activity of the cytochromes is relatively low.

Production of ATP. Bovarnick (10) used an ingenious method for the demonstration of ATP formation by rickettsiae respiring in the presence of glutamate. The test system consisted of inorganic phosphate, adenosine diphosphate (ADP), hexokinase, and glucose. Under these conditions ADP was phosphorylated to ATP, and glucose-6-phosphate was formed. The reaction required relatively large amounts of inorganic phosphate and ADP and was stimulated by the addition of small quantities of NAD and coenzyme A (CoA). Approximately 0.2 to 0.3 μmol of glucose-6-phosphate was formed for each atom of oxygen consumed. The reaction was completely inhibited by cyanide, indicating that a myokinase type enzyme, i.e., one converting two molecules of ADP to one of ATP and one of adenylic acid, was not involved. Dinitrophenol, as expected, inhibited phosphorylation in concentrations that did not interfere with respiration.

Admittedly, in the above-described experiments the rickettsiae were maintained in a highly artificial environment. In fact, the addition of ADP (or ATP) to glutamate reduced respiration by 30 to 40%, possibly because it interfered with the proper utilization of the corresponding endogenous factors. More satisfactory evidence of oxidative phosphorylation was obtained by Bovarnick and Allen (13) by the direct measurement of the ATP content of rickettsiae incubated under various conditions. Starved rickettsiae, namely rickettsiae incubated at 36 C for 3 h without substrate, contained no measurable ATP. When these starved rickettsiae were incubated with glutamate for 2.5 h at 30 C, the ATP level rose to 1.5 to 2.0 μmol per mg of rickettsial protein. When adenylic acid was also added, somewhat higher levels of ATP were obtained. The highest ATP levels demonstrated in rickettsiae were considerably lower than those generally encountered among viable bacteria. It is possible that rickettsiae are not capable of achieving an endogenous ATP level sufficiently high to sustain all their synthetic functions, and this may be one reason why they are incapable of independent existence.

Synthetic activities. It was shown by Bovarnick et al. (11, 18, 20) that rickettsiae are capable of synthesizing small amounts of protein and lipid. One of the simplest media that has supported protein synthesis is shown in Table 2. Synthesis was demonstrated by replacing one of the amino acids with one that was radioactively labeled and showing that it was incorporated into the trichloroacetic acid insoluble fraction. Incorporation was inhibited by chloramphenicol, which provided further evidence that it represented protein synthesis. To increase the sensitivity of the test, the labeled amino acid was added at a concentration lower than the rest, 0.005 to 0.01 instead of 0.1 mM. Also, low concentrations of rickettsiae (30 to 40 μg of protein per ml) were used because higher specific activities were obtained with these amounts.

It is not certain that all of the constituents listed in Table 2 are essential for optimal amino acid incorporation. However, the observations made on the effect of some of these compounds on incorporation (18) depicted better than any other experiments the physiology of rickettsiae, or at least of rickettsiae that had been subjected to the lengthy procedure of purification.

Optimal incorporation requires a K^+ concentration of at least 0.13 M. When all or a significant portion of the K salts are substituted by Na salts or sucrose, incorporation is greatly reduced. Mg^{2+} is an absolute requirement, whereas Mn^{2+} is not essential but stimulatory. There is good evidence that all of the natural amino acids must be present for the incorporation of one. Methionine-^{35}S, glycine-^{14}C (or glycine-2-^{14}C), or valine-1-^{14}C, used individually as the labeled amino acids, yielded comparable results. When all of the amino acids are omitted except the one that is labeled, incorporation is negligible. The same results are obtained when a single amino acid, such as serine, threonine, or valine, is omitted. One amino acid, glutamine, must be added in far larger concentrations than the others because it serves the dual function of providing energy as well as a source of glutamate for protein synthesis. Glutamine and glutamate can be used inter-

TABLE 2. *Composition of medium supporting amino acid incorporation in typhus rickettsiae*[a]

Constituent	Final concentration (mM)	Constituent	Final concentration (mM)
Salts		Amino acids—*Continued*	
KCl	160	L-isoleucine	0.20
K_2HPO_4	4.5	L-lysine	0.15
KH_2PO_4	0.8	L-methionine	0.05
$MgCl_2$	1.8	L-phenylalanine	0.08
$MnCl_2$	0.05	L-aspartic acid	0.10
$FeCl_2$	0.008	L-glutamine	5.0
Sodium acetate	1.2		
		Nucleotides and cofactors[b]	
Amino acids		ATP	1.0
DL-alanine	0.20	CMP	0.12
L-arginine	0.10	GMP	0.12
L-asparagine	0.10	UMP	0.12
L-cysteine	0.04	NAD	0.38
L-histidine	0.04	NADP	0.03
L-proline	0.10	CoA	0.038
L-hydroxyproline	0.10	Cocarboxylase	0.055
L-serine	0.10	Glutathione	0.5
L-threonine	0.15		
L-tryptophane	0.015	Protein (bovine plasma albumin or yolk sac protein)	0.25%
L-tyrosine	0.10		
L-valine	0.15		
Glycine	0.10	Indicator	
L-leucine	0.15	Phenol red	0.005%

[a] Adapted from Bovarnick and Schneider (18) on the basis of personal communication from Bovarnick.

[b] ATP, adenosine triphosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; UMP, uridine monophosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; CoA, coenzyme A.

changeably. An early report (20) that glutamate could not replace glutamine in supporting methionine-^{35}S incorporation was an error due to the contamination of glutamate with methionine, which reduced the specific activity of the radioactive label (18).

Despite the fact that glutamine, or glutamate, must be added as an energy-yielding substrate, rickettsiae must also be provided with exogenous ATP (18). This need can in part be met by the addition of ADP. Of course, it is not known to what extent this requirement reflects cell damage, but in any case it is an unusual bacterial requirement shared with *Chlamydia* (135) and only rarely and inconclusively described in other genera. In contrast to *Chlamydia*, rickettsiae need two sources of ATP, one to be generated endogenously from glutamine and one to be provided exogenously (18). This dual requirement suggests that certain functions are localized at the surface and others in the cytoplasm.

A large number of compounds were tested for their effect on incorporation. In addition to ATP, the other ribonucleotides are needed for maximal incorporation, whereas the deoxyribonucleotides do not affect it. NAD and reduced glutathione are essential. The effect of glutathione is so pronounced that, possibly, it does not act simply as a reducing agent but has cofactor function. Nicotinamide adenine dinucleotide phosphate (NADP), CoA, and cocarboxylase do not generally affect incorporation, but they are usually added because it was shown in other experiments that they increase rickettsial stability (18). Various mixtures of vitamins appeared to be without effect (Bovarnick, personal communication).

The incorporation medium must also contain a protein. Its chief function is undoubtedly to increase the stability of the rickettsiae. It may also play a specific role in metabolism by adsorbing critical nutrilites and, in some unknown manner, making them more readily available to the rickettsiae. Many natural proteins are toxic and cannot be used. Bovarnick found that only certain lots of bovine plasma albumin were satisfactory and obtained most

consistent results with a fraction of yolk sac protein extracted with acetone and ammonium sulfate (20).

In most of Bovarnick's experiments (18) incorporation was sustained for several hours. In general, the radioactivity in the trichloroacetic acid fraction was almost twice as high after 24 h of incubation as after 5 h. Maximal incorporation amounted to 10 μmol of amino acid per mg of rickettsial protein. A twofold increase in rickettsial protein would have required incorporation at least 100 times greater.

Bovarnick has also shown that rickettsiae synthesize lipids (11). Judging from the amount of acetate-*1*-^{14}C incorporated, lipid synthesis was minute, 0.25 μmol of acetic acid incorporated per mg of rickettsial protein with the more active preparations. However, it is probable that the total amount of lipid synthesized was much greater and that most of it was derived from acetyl-CoA produced from unlabeled glutamate (or glutamine) rather than from labeled acetate.

The energy requirements for lipid synthesis are similar to those described for protein synthesis. However, a mixture of amino acids, except for the energy-yielding glutamine or glutamate, is not required. Reduced pyridine nucleotides usually enhance incorporation (11).

It is difficult to assess the significance of the minute synthetic activities demonstrated with purified rickettsiae. Do they reflect the extent of the messenger RNA remaining with the organisms, the residual activities of enzymes which are derepressed only in an intracellular environment, or activities that can be further stimulated by greater wisdom in formulating the medium? Bovarnick compared rickettsiae to cell particulates rather than to intact cells and she was keenly aware of the fact that the rickettsiae used in her study had been injured during the process of purification and attributed her results, in part, to cell injury (18).

Physiology of cell injury. In a classic paper published in 1950, Bovarnick et al. (17) formulated a medium usually designated SPG, which favors the survival of a number of strains of rickettsiae. Its composition is based on the observations that rickettsiae are more stable in a sucrose than in a saline solution, in a saline solution high in K^+ rather than one high in Na^+, and in a pH close to 7.0 rather than 7.6. Also essential is the presence of a moderate amount of glutamate. The stability of rickettsiae is further increased by the addition of serum albumin.

This medium has been widely used for the storage of rickettsial suspensions in the frozen state, for their dilution before titration, and for other experimental procedures. Some more recent evidence indicates, however, that this medium may not be needed by intact cells, but compensates for cell damage and that, in some cases, it does not increase the stability of rickettsiae.

Myers et al. (79) studied the permeability of the cell membrane of *R. typhi* to sucrose and various electrolytes by phase-contrast microscopy observations of plasmolysis. The results clearly indicated that freshly harvested, unpurified rickettsiae had permeability properties very similar to those of *E. coli*. The cells were plasmolyzed by high concentrations of sucrose, NaCl, or KCl. This means that these compounds penetrated into the spaces between cell wall and cell membrane and did not cross into the cytoplasm, which shrank because of increased osmotic pressure. In contrast, when the rickettsiae were frozen and thawed or just subjected to a lengthy process of purification, the cells remained impermeable to sucrose but became permeable to the salts. These observations were confirmed by optical density measurements and radioisotope dilution techniques. Thus, it appears that the high K^+/Na^+ ratio required by purified rickettsiae is a reflection of lost ability to control their internal electrolyte ratio. Intact cells may not have this requirement.

Stoenner et al. (109) showed that spotted fever rickettsiae suspended in SPG lost about half of their viability during the brief period required to dilute them and to inject them into eggs. Rickettsiae suspended in phosphate-buffered saline did not suffer such a loss under the same conditions. These surprising results can best be interpreted by assuming that enhancement of catabolic activity by the glutamate of SPG does not always increase the stability of rickettsiae. Rees and Weiss (96) demonstrated that glutamate stabilized the metabolic activity of spotted fever rickettsiae most effectively in the presence of reduced glutathione and in a gas phase which contains very low levels of O_2. It is possible that in the experiments of Stoenner et al. (109) the redox potential was high enough to permit the accumulation of toxic levels of hydrogen peroxide. This is probably not an isolated incident since instances have been encountered in which glutamate is detrimental to typhus rickettsiae suspended in an unfavorable medium (14).

The foregoing examples serve to illustrate that most of the observations of the physiology

of rickettsiae have of necessity been made on damaged cells or cells maintained under unphysiological conditions. There are two types of cell damage that have been produced in the laboratory and studied extensively. One is the damage caused either by prolonged incubation of the rickettsiae at 0 C in saline solutions or by repeated freezing and thawing (12, 14). The other is starvation resulting from incubation of the rickettsiae without substrate at 36 C for several hours (13). Both types of damage are often compared to those produced on mitochondria or protozoa during procedures of isolation. As in the case of mitochondria, some of this damage can be prevented or repaired.

It was shown by Bovarnick et al. (15) that the survival of typhus rickettsiae, as measured by egg infectivity, mouse toxicity, hemolytic activity, and respiration, is increased in the presence of NAD. This suggests that rickettsiae slowly lose this coenzyme and that its presence in the medium prevents this loss. In fact, it was shown (12, 14) that when rickettsiae are suspended in a saline solution and frozen and thawed or simply maintained at 0 C for 18 h and then centrifuged, endogenous NAD no longer sediments with the cells but remains in the supernatant fluid, and the rickettsiae lose biological activity. In addition to NAD, rickettsiae probably lose other coenzymes such as CoA, and divalent ions, notably Mg^{2+}. If the damage is not too severe, rickettsiae can regain their biological activity when they are incubated with NAD, CoA, and divalent ions for 3 h at 33 C.

When rickettsiae are maintained at 36 C for several hours in the absence of substrate, loss of biological activity is accompanied by an almost complete disappearance of endogenous ATP (13). This loss can be prevented or restored by incubating the rickettsiae with glutamate or pyruvate. Added ATP, which is very effective in preventing loss of biological activity, can not always restore it.

The reaction of rickettsiae with exogenous ATP is a very curious one, as illustrated by the experiments of Bovarnick and Schneider (19). When the rickettsiae are freshly harvested from yolk sac, purified, and used immediately, they hemolyze sheep erythrocytes quite well in the presence of glutamate, but this reaction is strongly inhibited by exogenous ATP, and with ATP alone there is very little hemolysis. As the rickettsiae are progressively damaged, hemolysis with glutamate declines, ATP becomes less inhibitory, and hemolysis with ATP alone increases. Eventually rickettsiae are no longer able to hemolyze sheep erythrocytes in the presence of glutamate, but only in the presence of ATP.

It is not known to what extent the mechanism of cell damage and repair in rickettsiae differs from that of other bacteria. It is quite possible that the inability of rickettsiae to grow without host cells facilitated the studies of phenomena that may easily be overlooked in bacteria in which recovery is promptly followed by growth. It is tempting to postulate, however, that rickettsiae, unlike most other bacteria, must be able to interact with coenzymes and with other phosphorylated compounds within the host cell and that the above-described phenomena are just examples of such capabilities. Furthermore, there is good evidence that there are natural fluctuations in rickettsial virulence which involve loss and gain in known and unknown factors. This phenomenon has been encountered in the spotted fever rickettsiae and is usually called "reactivation".

Reactivation. It was shown by several of the early investigators of Rocky Mountain spotted fever that unfed adult *Dermacentor andersoni* ticks often contain rickettsiae that immunize guinea pigs but produce no recognizable illness. When ticks from the same batch are allowed to have a blood meal and the contents are injected into guinea pigs, a typical disease follows. Spencer and Parker (106) reproduced the same phenomenon in the laboratory by injecting ticks with virulent rickettsiae and storing them at refrigerator temperature for several months. As in the case of the natural infection, the rickettsiae remained immunogenic but were not virulent for guinea pigs, and virulence was fully reestablished when the ticks had a blood meal or were incubated at 37 C for 24 to 48 h.

Price (92, 93) obtained convincing evidence that this phenomenon could not be attributed to an increase in the number of rickettsiae or to a population shift. It was a true physiological change which Gilford and Price (40) were able to reproduce in vitro. They incubated infected yolk sac suspended in SPG at 25 C for 60 h, and showed that under these conditions rickettsiae lost their infectivity for eggs and guinea pigs. However, when 36 mM *p*AB was also added to the suspension, about 1% of the egg infectivity was retained but the rickettsiae were no longer virulent for guinea pigs. The addition of 15 mM *p*HB instead of *p*AB, or combinations of *p*AB and *p*HB, or *p*AB and 0.6 mM CoA resulted in retention of guinea pig virulence as well as egg infectivity. The lost guinea pig virulence of rickettsiae that were still infectious for eggs could be restored by incubation at 25 C for 6 h

with NAD or CoA, but not with *p*HB. Virulence was also restored by incubating the rickettsiae at 37 C for 24 h with suspensions of ground up adult ticks or nymphs that had received a blood meal. The factors in tick extracts responsible for this conversion have not been identified.

It is not known whether reactivation of spotted fever rickettsiae is analogous to the previously described reversible inactivation of typhus rickettsiae. These two phenomena have points of similarity but differ in one important respect. Typhus rickettsiae lose and regain all of their biological activities, including egg infectivity, at about the same rate, whereas spotted fever rickettsiae lose guinea pig virulence more rapidly than egg infectivity. Because a single egg passage will restore the guinea pig virulence of *R. rickettsi*, it is quite possible that the yolk sac of the chicken embryo offers to the spotted fever rickettsiae an especially favorable environment where activity is quickly restored and where partial cell damage can not be demonstrated. If this is the case, both phenomena entail the loss of important cofactors, but the steps involved in restoration of activity vary somewhat.

Metabolism of rickettsiae multiplying in host cells. Alexander (1, 2) has shown that cycloheximide, an inhibitor of eukaryotic but not of prokaryotic protein systems (34, 90), is an excellent tool for the study of the metabolism of chlamydiae multiplying in cell culture. This same compound has been used recently to study the independent metabolism of multiplying rickettsiae (127, 131). Four rickettsial species have been studied, namely *R. typhi*, *R. akari*, *R. rickettsi*, and *R. tsutsugamushi*. The host cell was the nonmultiplying (irradiated) L cell, except that irradiated duck embryo fibroblasts were used for *R. rickettsi*. The experimental design was a very simple one: the incorporation of a mixture of 15 ^{14}C-labeled amino acids or of ^{14}C-labeled adenine was measured at intervals in uninfected and infected cells both in the presence and absence of cycloheximide. The results clearly indicated that rickettsial multiplication was not affected by cycloheximide and that rickettsial infection elicited a highly significant increase in cycloheximide-resistant incorporation of amino acids and adenine into the trichloroacetic acid insoluble fractions. With *R. rickettsi* cycloheximide-resistant activity was small, possibly because this rickettsia never achieves a high density even within its most susceptible host cell. With the other three rickettsiae, at the peak of the infection cycloheximide-resistant activity reached 30 to 60% of the total. The above-described experiments leave little doubt that protein and nucleic acid syntheses accompanying rickettsial multiplication are of the prokaryotic type and that therefore these activities can be attributed to the rickettsiae.

Q Fever Rickettsiae

C. burneti is one of the sturdiest of the nonsporogenic organisms. When suspended in distilled water or sterile milk and stored at 4 C or room temperature, viability is retained for months and sometimes years. Flash pasteurization is not usually effective in sterilizing milk infected with *C. burneti* and there have been reports of incomplete inactivation of organisms maintained at 63 C for 40 min. The same is true of chemical sterilization. For example, 1% Formalin will inactivate *C. burneti* in 72 but not 24 h, and viability has been demonstrated after 4 days of storage at 4 C in 0.5% Formalin. *C. burneti* is not immediately inactivated by 5% acetic acid, 5% NaOH, or acetone (6, 95).

One would be led to believe, therefore, that *C. burneti* is ideally suited for the study of metabolic reactions and may well serve as the prototype of obligate intracellular bacteria. Unfortunately, nothing is further from the truth. Not only do intact resting cells display very little metabolic activity, but this activity is greatly affected by the composition of the suspending medium and by added cofactors (R. A. Ormsbee, personal communication). Although the optimal conditions for the demonstration of independent metabolic activity have not been elucidated, one must conclude that resting cells of *C. burneti* are relatively inert. Possibly, this characteristic, inability to interact with the environment unless conditions are favorable for growth, may be responsible for their high degree of stability.

The chief substrate of resting cells appears to be pyruvate, as shown by Ormsbee and Peacock (84). The rate, 0.25 μmol of O_2 consumed per h per mg of rickettsial protein, is about one-sixth of the rate of respiration of typhus rickettsiae in the presence of glutamate. Respiration of *C. burneti* can also be stimulated by oxaloacetate, succinate, serine, and as shown with considerable difficulty, by α-ketoglutarate, fumarate, and malate. Glutamate is utilized only in the presence of added NAD.

More rewarding have been the experiments with disrupted cells carried out by Paretsky and his collaborators (86). Many of these experiments included tests with intact cells which displayed very little, if any, activity. Paretsky,

on the strength of his investigations, came to the conclusion that Q fever rickettsiae have the principal physiological functions expected of bacteria. His findings do not explain why *C. burneti* can not be grown in a host cell-free medium.

Most surprising is the evidence that disrupted preparations of *C. burneti* have some enzymes of glucose metabolism, especially since the most sensitive methods have failed to demonstrate these activities with intact cells (85). The existence of intracellular hexokinase, glucose-6-phosphate, and 6-phosphogluconate dehydrogenases and more recently of aldolase activity and of other enzymes of the Embden-Meyerhof pathway have been demonstrated (29, 64, 65, 87). There is good evidence that Q fever rickettsiae have most, if not all, of the enzymes of the citric acid cycle. Paretsky and his co-workers (29, 88) have demonstrated the synthesis of citric acid from oxaloacetate and, to a smaller extent, from acetate and acetyl phosphate in the presence of NAD, CoA, and ATP. Oxidation of isocitrate, glutamate, and malate with reduction of their respective cofactors, NADP or NAD, has also been shown. It is not known whether glucose and citrate metabolism represent a true difference from events in typhus rickettsiae, since comparable experiments have not yet been done with the latter organisms.

In addition to the above-described reactions, which can be regarded as indicators of energy-yielding fuctions, Paretsky and his associates showed that disrupted Q fever rickettsiae have synthetic capabilities. Myers (75) demonstrated the presence of folic acid in *C. burneti*, and Mattheis et al. (71) isolated several types of folates, some with chromatographic elution patterns quite different from the folic acid derivatives of chicken embryos. These findings led to the investigation of reactions catalyzed by the folic acid series. Myers and Paretsky (78) showed that enzyme preparations of *C. burneti* can form serine from glycine and formaldehyde in the presence of tetrahydrofolic acid. These results, in turn, directed the attention of Mallavia and Paretsky (67) to two other one-carbon transfers, both involving carbamyl phosphate. They showed that citrulline was formed from ornithine and carbamyl phosphate and the reaction possibly proceeded to the synthesis of arginine. The pyrimide precursor ureidosuccinate was produced from aspartate and carbamyl phosphate in the presence of ATP.

Demonstration of synthesis of macromolecules was the next step. Mallavia and Paretsky (68) showed that cell-free preparations of *C. burneti* incorporated minute amounts of leucine, phenylalanine, and algal protein hydrolysate into hot trichloroacetic acid insoluble fractions. Evidence of a rickettsial DNA-dependent RNA polymerase was provided by Jones and Paretsky (52), who incubated rickettsial extracts with the four ribonucleoside triphosphates plus energy-generating compounds such as phosphoenolpyruvate and pyruvate kinase. When one of the four ribonucleoside triphosphates was labeled, it could be shown that it was incorporated into an insoluble trichloroacetic acid fraction. Incorporation was dependent on the presence of all four ribonucleosides and was enhanced by either heterologous or homologous DNA. It was inhibited by deoxyribonuclease or actinomycin D. The synthesized compound was destroyed by ribonuclease or dilute alkaline hydrolysis, and thus it was shown to have the properties of a polyribonucleotide.

Again, there is no reason to believe that *C. burneti* has enzyme systems not possessed by typhus rickettsiae. It just happened that early frustrations with disrupted typhus rickettsiae on the one hand and cell suspensions of Q fever rickettsiae on the other led investigators to different approaches and to findings of different significance.

WHY HAS INDEPENDENT CULTIVATION NOT BEEN ACHIEVED?

The question of failure to grow rickettsiae in a cell-free medium can be answered only in a highly speculative manner. Pertinent information, as we have seen in previous sections, is at best incomplete and in some cases misleading. Furthermore, any educated guessing must be based for the most part on negative results, and this is very dangerous. A single successful experiment may invalidate conclusions based on many well-planned and well-executed experiments in which rickettsiae have not grown. Speculations are necessary, however, because they constitute the substrate on which new experiments must feed. A current textbook incorrectly refers to rickettsiae as "tiny intracellular parasites," implying that the genome is too small to code for all functions necessary for independent existence. As we have seen, this hypothesis is not tenable (54). Some of the more obvious reasons why rickettsiae have not yet been cultivated in the absence of host cells are bravely discussed below.

Energy Parasitism

There is strong circumstantial evidence from the investigations carried out in the laboratories

of Weiss and of Moulder (72–74, 118, 135) that chlamydiae derive energy from the host cell. In sharp contrast, isolated rickettsiae derive ATP from the oxidation of glutamate (10), and this activity appears to be essential for penetration (27). This finding does not exclude the possibility that rickettsiae, once they are inside their host cells, are energy parasites. In fact, as discussed in a previous section, it was shown that for protein and lipid syntheses exogenous ATP must also be supplied (11, 18, 20). Although a requirement for exogenous ATP is rare among bacteria, it is not uncommon to find that separate energy-yielding reactions take place for transport and for synthetic activity (47, 69, 119). It is therefore conceivable that rickettsiae are capable of meeting their endogenous ATP requirements but depend on the host for energy for the transport of key metabolites. A critical experiment has probably not been performed which would exclude the possibility that ATP is a major requirement for the growth of isolated rickettsiae, since it is not known that all the other requirements have been met, and it can be assumed that in most experiments the cells were damaged to some extent. Bovarnick and Schneider (19) showed that the hemolytic activity of undamaged rickettsiae is inhibited by concentrations of ATP as low as 1 mM. The ideal experiment should contain a system generating ATP or other energy-yielding compounds at a low constant rate with maximal concentrations well below the toxic level.

Leakiness

The remarkable effects of the pyridine nucleotides and CoA on maintainance and restoration of activity of typhus and spotted fever rickettsiae, discussed in a previous section, were produced on damaged cells. It is not certain that intact cells require cofactors, although this is a distinct possibility, but it appears that their inclusion in the medium prevents their leakage from the cell. Both Moulder (74) and Hanks (45) have emphasized that a successful intracellular parasite must be able to release and accept numerous factors and create within the host cell a microenvironment in which the required metabolites are in proper balance. In an extracellular environment this "leakage" may be a one-way street and may lead to the steady deterioration of the rickettsiae. As we have seen, various factors, such as moderately high osmolarity, a high K^+/Na^+ ratio, sucrose, and serum albumin, retard leakage and stabilize rickettsiae, but these factors may not be adequate when the volume is disproportionate to the number of rickettsiae. If "leakiness" is indeed the major factor in frustrating extracellular rickettsial growth, the ideal experiment must take into consideration the maximal volume of medium that a given number of rickettsiae can tolerate. The extraordinary stability of *C. burneti* may indicate that leakage does not occur with this microorganism or that the lost compounds can be efficiently reacquired in an intracellular environment. There is no experimental evidence yet to distinguish between these two possibilities.

Unidentified Auxotrophy

The record of the attempts that have been made to grow rickettsiae on established or experimental cell-free media is not complete. All of these experiments have been, of course, unsuccessful, and most of them have not been published. For example, the work of Bovarnick on protein and lipid syntheses (11, 18, 20) in complex media was the result of frustration over attempts to cultivate the organisms in these and other media. There is always the possibility that in each instance a key metabolite was not added. The search for this metabolite must continue, but one must keep in mind that compounds that are not needed are often toxic or antagonistic. This is particularly true of natural products such as tissue extracts. A medium containing a judicious but conservative number of defined chemicals is more likely to be successful than one that is too generous.

Highly Sensitive Regulatory Mechanism

Extracellular rickettsiae are probably analogous to resting bacteria and display limited enzymatic activities. A suitable environment may be required for the induction of some of their synthetic enzymes. It is conceivable that the balance between repression, induction, and end-product inhibition is more delicate in rickettsiae than in other bacteria and that it is exquisitely adapted to the intracellular environment. The evolutionary advantage of a sensitive balance is obvious. The rickettsiae would be able to multiply only when conditions are right in an intracellular environment. As multiplication progresses and some of the host-cell metabolites become scarcer, induction would stop, or end-product inhibition would start, before the host cell is irreparably damaged. This is actually the situation in many relationships between rickettsiae and their natural host cells. In the laboratory we strive to produce as many rickettsiae as possible and we disrupt this delicate balance. We select a host cell that can induce the synthetic enzymes of the rickettsiae but sends unclear signals for inhibition until infection proceeds to the total destruction of the host cell. The resulting rickettsiae most likely have

enzyme systems which have been abruptly repressed. If this is indeed the reason why rickettsiae have not been cultivated in host cell-free medium, it represents a serious obstacle. Information on mechanisms of enzyme regulation in rickettsiae is urgently needed.

Comment

It would be foolhardy to rank the above-listed reasons for failure to cultivate rickettsiae independently of their host cells in order of probability. Attention must be paid to all of these possibilities. A brilliant new idea for the growth of rickettsiae will not bear fruit if all of the other requirements are not adequately met. It can not be overemphasized that all work should be done with rickettsiae damaged as little as possible. It might be well to assume that any rickettsia artificially separated from host cells has suffered some damage and should be treated with the care usually accorded to protoplasts or mitochondria. This applies also to *C. burneti*, whose enormous ability to remain infectious may belie the damage that might have been inflicted to its metabolic capabilities in an extracellular environment.

The reader may be tempted to ask to what extent the successful cultivation of *Rochalimaea quintana* on blood agar (115) can pave the way for the independent growth of rickettsiae. *R. quintana* is extracellular in its natural host vector and possibly in man, and it grows with difficulty in eggs or tissue culture. Its most notable requirement for growth is hemin (77), which probably is not needed by rickettsiae. With the benefit of hindsight, one may say that the cultivation of *R. quintana* on blood agar was not surprising and contributed little to the solution of our problem of cultivating rickettsiae without host cells. We can learn a great deal from basic studies of many nutritionally fastidious bacteria or auxotrophic mutants and by keeping faith that the tenets of comparative biochemistry apply also to rickettsiae.

A FINAL REMARK

A review usually deals with a field that is rapidly expanding. This review deals with an area of microbiology which at best has received only moderate attention in recent years. Half of the publications quoted here were written more than 10 years ago. As a result, some of the concepts and techniques described are obsolete and much of the information required for a comprehensive discussion of the biology of rickettsiae is lacking. This waning interest may in part stem from a dubious sense of security that rickettsial infections no longer pose grave public health problems, but more importantly it also derives from the misconception that rickettsiae have little to offer to the student of basic biology or that the efforts needed for meaningful information are hardly worth the expected results. Neither attitude can be well defended. Modern technology is providing numerous new avenues of approach to the study of these microorganisms which have evolved what appears to be a most ingenious mechanism of interaction with their microenvironment. Our reticence to work with rickettsiae is no longer justified.

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